Analysis of opossum kidney NaPi-IIc sodium-dependent phosphate transporter to understand Pi handling in human kidney

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Abstract

Background The role of Na^+-dependent inorganic phosphate (Pi) transporters in the human kidney is not fully clarified. Hereditary hypophosphatemic rickets with hypercalciuria (HHRH) is caused by loss-of-function mutations in the IIc Na^+-dependent Pi transporter (NPT2c/Npt2c/NaPi-IIc) gene. Another Na^+-dependent type II transporter, (NPT2A/Npt2a/NaPi-IIa), is also important for renal Pi reabsorption in humans. In mice, Npt2c deletion does not lead to hypophosphatemia and rickets because Npt2a compensates for the impaired Pi reabsorption. To clarify the differences between mouse and human, we investigated the relation between NaPi-IIa and NaPi-IIc functions in opossum kidney (OK) cells.

Methods We cloned NaPi-IIc from OK cells and created opossum NaPi-IIc (oNaPi-IIc) antibodies. We used oNaPi-IIc small interference (si)RNA and investigated the role of NaPi-IIc in Pi transport in OK cells.

Results We cloned opossum kidney NaPi-IIc cDNAs encoding 622 amino acid proteins (variant1) and examined their pH- and sodium-dependency. The antibodies reacted specifically with 75-kDa and 150-kDa protein bands, and the siRNA of NaPi-IIc markedly suppressed endogenous oNaPi-IIc in OK cells. Treatment with siRNA significantly suppressed the expression of NaPi-4 (NaPi-IIa) protein and mRNA. oNaPi-IIc siRNA also suppressed Na^+/H^+ exchanger regulatory factor 1 expression in OK cells.

Conclusion These findings suggest that NaPi-IIc is important for the expression of NaPi-IIa (NaPi-4) protein in OK cells. Suppression of Npt2c may downregulate Npt2a function in HHRH patients.

Introduction

Inorganic phosphate (Pi) retention initiates the development of many complications in chronic kidney disease, such as secondary hyperparathyroidism and bone and cardiovascular diseases [1, 2]. The kidneys play an important role in inorganic phosphate (Pi) retention [1, 2]. Type II sodium-dependent Pi cotransporters (SLC34A1/NPT2A/Npt2a/NaPi-IIa and SLC34A3/NPT2C/Npt2c/NaPi-IIc) are expressed in the renal proximal tubular cells [3, 4]. Phosphaturic factors such as fibroblast growth factor 23/klotho and parathyroid hormone (PTH) suppress both transporters [3, 4]. About 85% to 90% of filtered Pi is reabsorbed in the renal proximal tubule, mainly by the NaPi-IIa cotransporter in rodents [3, 4].

The biochemical features of Npt2a knockout mice resemble those of patients with hereditary hypophosphatemic rickets with hypercalciuria (HHRH), which is an autosomal recessive form characterized by reduced renal phosphate reabsorption, hypophosphatemia, and rickets [5, 6]. No mutations of the Npt2a gene are detected, however, in HHRH patients [7]. In contrast, several groups have identified that human NaPi-IIc mutations cause HHRH [8-10]. Individuals with HHRH carry compound heterozygous or homozygous (comp/hom) loss-of-function mutations in the NPT2c gene [8-10]. These mutations result in urinary Pi wasting and hypophosphatemic rickets, bowing, and short stature, as well as elevated $1,25(OH)_2D$ levels [8-10]. These reports support the view that NaPi-IIc is a major Pi transporter in the kidney [3, 4].

NaPi-IIa (SLC34A1) mutations have been identified by several laboratories investigating adult patients with kidney stones and reduced bone density or pediatric patients with hypophosphatemia and hyperphosphaturia, infantile idiopathic hypercalciuria, or nephrocalcinosis and kidney stones [11-14]. These findings support the idea that NaPi-IIa is also important for renal Pi reabsorption.

We previously cloned Npt2c (SLC34A3) from human and rodent kidneys [15]. Npt2c, like Npt2a, is expressed in the brush border membrane (BBM) of the proximal renal tubular cells and is subject to similar endocrine regulation [3, 4]. In animal studies, Npt2c defects do not cause hypophosphatemia and rickets/osteomalacia, while Npt2a/Npt2c double knockout mice exhibit hypophosphatemia, severe rickets, and osteomalacia, suggesting that Npt2a compensates for Npt2c function [16-18]. In humans, however, Npt2a might not compensate for Npt2c defects [3, 4, 8-10] . To evaluate interactions between NaPi-IIa and NaPi-IIc in human kidney proximal tubular cells, we investigated human, rat, mouse proximal tubular cells. We were unable to detect NaPi-IIc protein, however, and therefore used other cultured cells to study NaPi-IIc protein.

The OK cells were originally cloned from *Didelphis virginiana* [19]. More recently, Eshbach et al characterized the *D. virginiana* OK cell transcriptome via de novo transcriptome assembly [20]. The OK cell transcriptome assembly is most closely related to the human proximal tubule based on a curated list of proximal tubule-specific functional genes in humans [20]. There is no information on the NaPi-IIc gene and its regulatory mechanisms in OK cells. In the present study, to understand HHRH pathology, we cloned NaPi-IIc and investigated the roles of NaPi-IIc in OK cells.

Materials and Methods

Cloning of D. virginiana (OK cells) NaPi-IIc

Total RNA from OK cells was prepared using ISOGEN (Nippon Gene, Tokyo, Japan) [21]. The genetic information of *Monodelphis domestica* (opossum), but not that of *D. virginiana,* has been reported. cDNA fragments were synthesized using the Moloney murine leukemia virus, reverse transcriptase (Superscript; Invitrogen, Carlsbad, CA), and Oligo (dT) 12-18 Primer, or a specific reverse transcription primer designed based on the *M. domestica*

NaPi-IIc sequence for 5'-RACE (Table 1), and then 5'-RACE was performed using a 5'-Full RACE core Set (TaKaRa, Shiga, Japan) and 3'-RACE reaction [22] to obtain the 5'- and 3'- sequences of *D. virginiana* NaPi-IIc. After the 5'- and 3'- sequence determination, full-length *D. virginiana* NaPi-IIc was subcloned into pBluescript II SK (-) (Stratagene, La Jolla, CA), and sequence analysis was performed to determine the full-length sequence of *D. virginiana* NaPi-IIc (variant1 accession number: LC389083).

Plasmid constructs

Full-length wild-type (WT) human NaPi-IIc, mouse NaPi-IIc, and *D. virginiana* NaPi-IIc with a FLAG tag (pCMV-Tag2A vector; Stratagene; FLAG-NaPi-IIc) at the amino terminus were generated using standard cloning techniques [23, 21].

siRNA constructs

All siRNA duplexes were purchased from Sigma-Aldrich (St. Louis, MO), Nippon Gene, or Invitrogen. Double-stranded *D. virginiana* NaPi-IIc (oNaPi-IIc) and NaPi-IIa (oNaPi-IIa) siRNA constructs were designed by oNaPi-IIc and oNaPi-IIa (GenBank Sequence; L26308.1) mRNA sequencing, respectively. The siRNA sequences are listed in Table 2.

Cell culture and plasmid transfection

OK cells were obtained from American Type Culture Collection (ATCC; Rockville, MD) and 3B/2 clones were a kind gift from Dr. J. Biber (Zurich University, Switzerland). The OK cells were maintained in an appropriate

medium [23]. siRNA transfections were performed using LipofectAMINE® RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Quantitative polymerase chain reaction analysis

Quantitative polymerase chain reaction (PCR) was performed using StepOnePlus™ (Applied Biosystems, Foster City, CA). The reaction mixture comprised 10 μl SYBR Premix Ex Taq (Perfect Real Time; TakaRa) with specific primers (Table 3). The PCR reactions were initiated with denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 15 s. Data were evaluated with SDS v. 1.2×with RQ software.

Pi uptake studies in OK cells

Pi transport was studied in monolayers of OK cells transfected with each siRNA in 12-well plates. Uptake experiments were performed as previously described [21, 24].

Xenopus laevis oocyte expression and Pi uptake studies

cRNAs obtained by in vitro transcription using T7 RNA polymerase for oNaPi-IIc cDNA in plasmid vector pCMV-Tag2A linearized with Kpn I as described previously [25]. Xenopus oocyte expression studies and uptake measurements were performed as described previously [15]. The uptake rates of ^{32}P were measured 2–3 days after injecting the cRNA [15].

Preparation of rabbit polyclonal anti-oNaPi-IIc, oNaPi-IIa, and human NaPi-IIa and NaPi-IIc antibodies

Amino acid peptide sequences from the amino-terminus (RTEDAGMEEFRTQVPSWSPC; amino acid (aa) position 22-40, or CKTSGQSWKELSRAGRIRR; aa position 57-74,) and from the C-terminal (PRAHDHEDCAEPV; aa position 592-605) of oNaPi-IIc were fused to keyhole limpet hemocyanin and used to generate rabbit polyclonal antibody. For opossum NaPi-4 (oNaPi-IIa), a peptide identical to the carboxy-terminal 12-aa sequence of NaPi-4 (LGVLSQHNATRL) was generated as described previously [21, 24].

In addition, an amino acid peptide sequence from the amino-terminus (CYENPEILASQQL; aa position 587-599) of human NaPi-IIc was used to generate rabbit polyclonal antibody by Sigma-Aldrich. We used the specific antibodies (C-terminal peptide LALPAHHNATRL) for detection of human, rat and mouse NaPi-IIa protein as described previously [26].

Confocal microscopy analysis

Cells were imaged using an A1R confocal laser scanning microscope system (Nikon, Tokyo, Japan) equipped with a 60×oil immersion objective. Immunostaining was performed as described previously [21, 24].

Preparation of BBMVs and whole homogenate for immunoblotting

BBMVs were prepared from the kidneys from the mice using the Ca^{2+} precipitation method as previously described [16].

Immunoblot analysis

Cells grown on 6-well plates were transfected as described previously and above [21, 24]. We used anti-oNaPi-IIc polyclonal (1:2000), anti-oNaPi-IIa polyclonal (1:2000), anti-NHE3 monoclonal (a kind gift from Dr. O. Moe,

University of Texas Southwestern Medical Center, Dallas, Texas; clone 3H3, 1:8), anti-Megalin polyclonal (a kind gift from Dr. A. Saito, Niigata University, Niigata, Japan ; 1 : 1000), anti-Na^+/K^+-ATPase α1 monoclonal (Merck Millipore; 05-369, 1:5000), anti-villin monoclonal (Merck Millipore; MAB1671, 1:1000), anti-NHERF-1 polyclonal (LS BioSciences, WA; LS-C46891, 1:1000), and anti-actin monoclonal antibodies (Chemicon, Temecula, CA; used as an internal control, 1:10000), in 5% skim milk in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20 (TBST), followed by treatment with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA; 1:10000). Signals were detected using the Immobilon Western detection system (Millipore).

Human kidney samples

Human kidney fractions were prepared from normal adult human kidneys obtained from National Disease Research Interchange (NDRI, Philadelphia, PA) and stored frozen at -80℃. Research involving human tissue was approved by the institutional review board of Tokushima University Hospital and the NDRI obtained informed consent from donors of human tissue. Tissue from adult kidney cortex (No. 111 or No. 112) was minced, then homogenized in the homogenate buffer as described previously [27].

Statistical analysis

Data are expressed as means ± SE. Differences among multiple groups were analyzed by ANOVA followed by Scheffe's test. The significance of differences between two experimental groups was established by ANOVA followed by Student's t test. A P value of less than 0.05 was considered significant.

Results

Characterization of antibodies against opossum NaPi-IIc

We cloned two variants of opossum kidney NaPi-IIc cDNAs (oNaPi-IIc) (*D. virginiana*) that were 622 and 602 amino acids long (Fig. 1). We aligned these variants with the full-length amino acid sequence of *M. domestica*. oNaPi-IIc of OK cells (*D. virginiana* type) exhibited 93.3% of homology with the *Monodelphis* type. The full-length amino acid sequence of oNaPi-IIc cotransporters in opossum (NCBI Reference Sequence, XP_007475507.1) and human (NP_001170787.1) had 69% homology (Fig. 1). Opossum NaPi-IIc and mouse NaPi-IIc had 68% homology. In addition, mouse and human NaPi-IIc had 80% homology.

oNaPi-IIc expression in OK cells

oNaPi-IIc mRNA levels were significantly increased at day 4 and even further increased at day 6 (Fig. 2A). The oNaPi-IIa, NHE3, 24 hydroxylase (24OHase), and Megalin mRNA levels were significantly increased at 3, 4, and 6 days, and Pit-1 mRNA levels were significantly decreased (Suppl Figure 1). In addition, we created specific antibodies against oNaPi-IIc protein (N-terminal antibodies, N-1, N-2; C-terminal antibodies, C-1; Fig. 2B, C). Only the N-terminal antibodies (N-1) were clearly reacted with the ~75-kDa protein in OK cell lysate (Fig. 2C). In transfected FLAG-oNaPi-IIc in OK cells, the N-1 antibodies clearly reacted with the ~150kDa and ~75 kDa bands (Fig. 2C). In the competition assay using antibodies incubated with antigen peptide (N-1), the 150-kDa and 75-kDa bands were completely abolished (Fig. 2D). Both protein bands were also detected by FLAG antibodies (Fig. 2E). Two prominent protein bands of NaPi-IIc, similar in size to those in human and mouse NaPi-IIc, were detected in OK cells (Fig. 2E). An oNaPi-IIc–reactive band was detected at ~75 kDa in differentiated OK cells at 4 and 6 days

(Fig. 2F). N-terminal specific antibodies (N-1) were detected in the exogenously expressed oNaPi-IIc in apical patches in the OK cells (Fig. 2G).

Functional analysis of oNaPi-IIc transporter

We investigated whether the cloned oNaPi-IIc functions in sodium-dependent Pi cotransport activity (Fig. 3). Injection of oNaPi-IIc cRNA into Xenopus oocytes markedly increased oNaPi-IIc–dependent NaPi cotransport activity in the presence of Na ions (Fig. 3A, B). The pH-dependent NaPi cotransport activity (pH5.5-pH 8.5) was maximal at pH 8.5 (Fig. 3C). In addition, the Km for Pi was 81.4 μM (Fig. 3D). These characteristics (Km and pH dependency) of oNaPi-IIc are similar to those of human and mouse NaPi-IIc transporters.

Effect of oNaPi-IIc siRNA on membrane proteins in OK cells

We investigated the effect of oNaPi-IIc siRNA on mRNA and protein levels in OK cells (Fig. 4A). Treatment with the oNaPi-IIc siRNA markedly decreased oNaPi-IIc mRNA and protein levels in OK cells (Fig. 4B, C).

In OK cells treated with oNaPi-IIc siRNA (20 or 50 pmol/ml), the protein levels of NaPi-IIa, NHE3 (20 pmol/ml only), and NHERF-1 were significantly decreased (Fig. 4D, E). In contrast, Na^+/K^+-ATPase protein levels were markedly increased in oNaPi-IIc siRNA-treated OK cells. These observations indicate that oNaPi-IIc downregulation affects NaPi-IIa, NHE3, and NHERF-1 protein levels. NaPi-IIa mRNA levels were significantly reduced (Fig. 4F), but NHE3, Megalin, and NHERF-1 (data not shown) mRNA levels were not. The oNaPi-IIc siRNA treatment markedly reduced Na^+-dependent Pi cotransport activity in OK cells (Fig. 4G). These observations suggest that suppressing endogenous oNaPi-IIc affects Na^+-dependent Pi cotransport activity (56% reduction

compared with the control). Levels of proliferating cell nuclear antigen (PCNA), a growth marker, did not differ between siRNA-treated and control OK cells (data not shown).

In addition, NaPi-IIa siRNA treatment of OK cells suppressed NaPi-4 mRNA and protein levels (data not shown) and reduced Na+-dependent Pi cotransport activity to ~50% that of the control (scramble siRNA) (Fig. 4G). These data clearly indicate that oNaPi-IIc and oNaPi-IIa siRNA affects Na^+-Pi transport activity.

Effect of siRNAs on dome formation in OK cells

Reducing sodium transport affects dome formation in renal epithelial cells [28]. NHE3, NHERF-1, NaPi-IIa, and Na^+/K^+-ATPase are involved in sodium transport in OK cells (Fig. 5). Dome formation in OK cells was significantly inhibited by treatment with oNaPi-IIc siRNA (Fig. 5A, B), whereas dome formation was only slightly inhibited by treatment with NaPi-IIa siRNA at 4 days, and not at all at 6 days (Fig. 5B). Furthermore, phosphonoformic acid (type II Na-Pi transporter Npt2a and Npt2c inhibitor) treatment decreased dome formation in OK cells at 7 days (Fig. 5C). Reduced dome formation is due to the suppression of oNaPi-IIc, and not to Na^+-dependent Pi transport activity.

NaPi-IIa and NaPi-IIc protein in Npt2c knockout mouse kidney and human adult kidney

We further investigated the levels of NaPi-IIa and NHERF-1 protein in Npt2c knockout ($Npt2c^{-/-}$) mice. NaPi-IIa and NHERF-1 protein levels did not differ between the $Npt2c^{-/-}$ and control mice (Fig. 6A). Analysis of human adult normal kidney samples from NDRI revealed NaPi-IIa and NaPi-IIc protein in the kidney homogenate (Fig. 6B).

Discussion

In the present study, we cloned oNaPi-IIc and analyzed its role in OK cells. oNaPi-IIc is 622 amino acids long and functional analysis revealed that the Km for Pi and the pH and sodium-dependency were similar to those of human and mouse NaPi-IIc. NaPi-IIc siRNA treatment suppressed the expression of NaPi-IIa (NaPi-4) protein and mRNA and decreased NHERF-1 protein levels. We suggest that in OK cells, NaPi-IIc (Npt2c) deficiency leads to NaPi-IIa (Npt2a) suppression.

The most important finding of this study is that the cause of HHRH can be clearly attributed to Pi transport abnormalities in the kidney. The original description of NaPi-IIc suggests that this transporter is exclusively expressed in mouse and rat kidney [25, 15]. Analyses of public RNAseq expression databases and qRT-PCR tissue expression, however, suggest that many tissues express low levels of NaPi-IIc [29, 30]. Furthermore, a kidney-specific and inducible NaPi-IIc-deficient mouse model (CKO) exhibits no Pi abnormality because renal NaPi-IIa can compensate for the loss of NaPi-IIc function in this model [18]. The importance of the widespread expression of NaPi-IIc outside of the kidneys for human Pi metabolism remains unknown [31]. In particular, expression of NaPi-IIc in bone may be involved in HHRH pathology. The findings of the present study, however, suggest that NaPi-IIc deficiency affects the expression of NaPi-IIa in OK cells. If NaPi-IIc also regulates the expression of NaPi-IIa in human kidney, the cause of the pathology in HHRH is Pi transport abnormalities in the kidney, and not bone.

The mechanism by which Npt2c deletion suppresses Npt2a expression is thought to be important for understanding HHRH. In the present study, we found that NHERF-1 protein levels were significantly decreased in OK cells by treatment with NaPi-IIc siRNA. NHERF-1 is essential for the positioning of NaPi-IIa in OK cells [32, 33]. It is possible that a reduction of NHERF-1 leads to alterations in NaPi-IIa protein levels. In addition, siRNA

oNaPi-IIc downregulates NaPi-IIa mRNA in OK cells. More recently, Clark et al reported that NHERF-1 functions to control NaPi-4 (NaPi-IIa) mRNA stability in OK cells [34]. NHERF-1 helps maintain steady-state NaPi-IIa mRNA levels in OK cells through indirect mechanisms that facilitate promoter protein-DNA interactions at the NaPi-4 proximal promoter [34]. Indeed, Karim et al identified mutations of NHERF-1 in patients with hypophosphatemia, low TmP/GFR, and normal serum PTH and FGF23 concentrations [35]. In addition, the NHERF-1-NaPi-IIa interaction is essential for controlling NaPi-IIa expression at the plasma membrane in human kidney [32, 36].

In the present study, we detected NaPi-IIa protein expression in human kidney samples. Even in a state of impaired NaPi-IIc function, it appears that NaPi-IIa supports the Pi reabsorption defect in human kidney, as observed in Npt2c knockout mice. In this context, the decline of NaPi-IIc may affect NaPi-IIa function in the human kidney. These data suggest that serum Pi levels in humans and mice are regulated by different transport systems. Serum Pi levels (8-10 mg/dl) are highly maintained in rodents compared with humans (3-5 mg/dl) and opossum (5-7 mg/dl) [37, 38]. The different stoichiometries between NaPi-IIa (electrogenic transport) and NaPi-IIc (electroneutral transport) indicate that the theoretical Pi concentrating capacity is approximately 100-fold higher for NaPi-IIa than for NaPi-IIc [4]. In humans and opossum, NaPi-IIc may regulate the expression of NaPi-IIa to maintain low plasma Pi levels. In contrast, in mice, NaPi-IIa mainly functions to maintain a high plasma Pi concentration. In fact, mouse Npt2c is necessary only during the growth phase [15].

Finally, the present study demonstrated that oNaPi-IIc is essential for NaPi-4 (NaPi-IIa) expression in OK cells. Unlike the role of NaPi-IIc in mouse kidney, NaPi-II transporters may provide mutual support in human and opossum kidney. Further studies are needed to clarify the mechanisms of NaPi-4 downregulation in OK cells.

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Conflict of interest

Authors have declared that no conflict of interest exists.

Human and Animal Right

Mice were handled in accordance with the Guidelines for Animal Experimentation of Tokushima University School of Medicine (T29-3). This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Tokushima University Hospital (1343-1).

References

1. Block GA, Ix JH, Ketteler M, Martin KJ, Thadhani RI, Tonelli M et al. Phosphate homeostasis in CKD: report of a scientific symposium sponsored by the National Kidney Foundation. American journal of kidney diseases : the official journal of the National Kidney Foundation. 2013;62:457-73

2. Vervloet MG, Sezer S, Massy ZA, Johansson L, Cozzolino M, Fouque D et al. The role of phosphate in kidney disease. Nature reviews Nephrology. 2017;13:27-38

3. Miyamoto K, Haito-Sugino S, Kuwahara S, Ohi A, Nomura K, Ito M et al. Sodium-dependent phosphate cotransporters: lessons from gene knockout and mutation studies. Journal of pharmaceutical sciences. 2011;100:3719-30

4. Wagner CA, Hernando N, Forster IC, Biber J. The SLC34 family of sodium-dependent phosphate transporters. Pflugers Archiv : European journal of physiology. 2014;466:139-53

5. Tieder M, Modai D, Samuel R, Arie R, Halabe A, Bab I et al. Hereditary hypophosphatemic rickets with hypercalciuria. The New England journal of medicine. 1985;312:611-7

6. Beck L, Karaplis AC, Amizuka N, Hewson AS, Ozawa H, Tenenhouse HS. Targeted inactivation of Npt2 in mice leads to severe renal phosphate wasting, hypercalciuria, and skeletal abnormalities. Proceedings of the National Academy of Sciences of the United States of America. 1998;95:5372-7

7. Jones A, Tzenova J, Frappier D, Crumley M, Roslin N, Kos C et al. Hereditary hypophosphatemic rickets with hypercalciuria is not caused by mutations in the Na/Pi cotransporter NPT2 gene. Journal of the American Society of Nephrology : JASN. 2001;12:507-14

8. Lorenz-Depiereux B, Benet-Pages A, Eckstein G, Tenenbaum-Rakover Y, Wagenstaller J, Tiosano D et al. Hereditary hypophosphatemic rickets with hypercalciuria is caused by mutations in the sodium-phosphate cotransporter gene SLC34A3. American journal of human genetics. 2006;78:193-201

9. Bergwitz C, Roslin NM, Tieder M, Loredo-Osti JC, Bastepe M, Abu-Zahra H et al. SLC34A3 mutations in patients with hereditary hypophosphatemic rickets with hypercalciuria predict a key role for the sodium-phosphate cotransporter NaPi-IIc in maintaining phosphate homeostasis. American journal of human genetics. 2006;78:179-92

10. Yamamoto T, Michigami T, Aranami F, Segawa H, Yoh K, Nakajima S et al. Hereditary hypophosphatemic rickets with hypercalciuria: a study for the phosphate transporter gene type IIc and osteoblastic function. Journal of bone and mineral metabolism. 2007;25:407-13

11. Prie D, Huart V, Bakouh N, Planelles G, Dellis O, Gerard B et al. Nephrolithiasis and osteoporosis associated with hypophosphatemia caused by mutations in the type 2a sodium-phosphate cotransporter. The New England journal of medicine. 2002;347:983-91

12. Rajagopal A, Braslavsky D, Lu JT, Kleppe S, Clement F, Cassinelli H et al. Exome sequencing identifies a novel homozygous mutation in the phosphate transporter SLC34A1 in hypophosphatemia and nephrocalcinosis. The Journal of clinical endocrinology and metabolism. 2014;99:E2451-6
13. Schlingmann KP, Ruminska J, Kaufmann M, Dursun I, Patti M, Kranz B et al. Autosomal-Recessive Mutations in SLC34A1 Encoding Sodium-Phosphate Cotransporter 2A Cause Idiopathic Infantile Hypercalcemia. Journal of the American Society of Nephrology : JASN. 2016;27:604-14
14. Dinour D, Davidovits M, Ganon L, Ruminska J, Forster IC, Hernando N et al. Loss of function of NaPiIIa causes nephrocalcinosis and possibly kidney insufficiency. Pediatric nephrology. 2016;31:2289-97
15. Segawa H, Kaneko I, Takahashi A, Kuwahata M, Ito M, Ohkido I et al. Growth-related renal type II Na/Pi cotransporter. The Journal of biological chemistry. 2002;277:19665-72
16. Segawa H, Onitsuka A, Kuwahata M, Hanabusa E, Furutani J, Kaneko I et al. Type IIc sodium-dependent phosphate transporter regulates calcium metabolism. Journal of the American Society of Nephrology : JASN. 2009;20:104-13
17. Segawa H, Onitsuka A, Furutani J, Kaneko I, Aranami F, Matsumoto N et al. Npt2a and Npt2c in mice play distinct and synergistic roles in inorganic phosphate metabolism and skeletal development. American journal of physiology Renal physiology. 2009;297:F671-8
18. Myakala K, Motta S, Murer H, Wagner CA, Koesters R, Biber J et al. Renal-specific and inducible depletion of NaPi-IIc/Slc34a3, the cotransporter mutated in HHRH, does not affect phosphate or calcium homeostasis in mice. American journal of physiology Renal physiology. 2014;306:F833-43
19. Koyama H, Goodpasture C, Miller MM, Teplitz RL, Riggs AD. Establishment and characterization of a cell line from the American opossum (Didelphys virginiana). In vitro. 1978;14:239-46
20. Eshbach ML, Sethi R, Avula R, Lamb J, Hollingshead DJ, Finegold DN et al. The transcriptome of the Didelphis virginiana opossum kidney OK proximal tubule cell line. American journal of physiology Renal physiology. 2017;313:F585-F95
21. Ito M, Sakurai A, Hayashi K, Ohi A, Kangawa N, Nishiyama T et al. An apical expression signal of the renal type IIc Na+-dependent phosphate cotransporter in renal epithelial cells. American journal of physiology Renal physiology. 2010;299:F243-54
22. Scotto-Lavino E, Du G, Frohman MA. 3' end cDNA amplification using classic RACE. Nature protocols. 2006;1:2742-5
23. Ito M, Iidawa S, Izuka M, Haito S, Segawa H, Kuwahata M et al. Interaction of a farnesylated protein with renal type IIa Na/Pi co-transporter in response to parathyroid hormone and dietary phosphate. The Biochemical journal. 2004;377:607-16

24. Haito-Sugino S, Ito M, Ohi A, Shiozaki Y, Kangawa N, Nishiyama T et al. Processing and stability of type IIc sodium-dependent phosphate cotransporter mutations in patients with hereditary hypophosphatemic rickets with hypercalciuria. American journal of physiology Cell physiology. 2012;302:C1316-30
25. Ohkido I, Segawa H, Yanagida R, Nakamura M, Miyamoto K. Cloning, gene structure and dietary regulation of the type-IIc Na/Pi cotransporter in the mouse kidney. Pflugers Archiv : European journal of physiology. 2003;446:106-15
26. Katai K, Segawa H, Haga H, Morita K, Arai H, Tatsumi S et al. Acute regulation by dietary phosphate of the sodium-dependent phosphate transporter (NaP(i)-2) in rat kidney. Journal of biochemistry. 1997;121:50-5
27. Kimura H, Murad F. Localization of particulate guanylate cyclase in plasma membranes and microsomes of rat liver. The Journal of biological chemistry. 1975;250:4810-7
28. Su HW, Yeh HH, Wang SW, Shen MR, Chen TL, Kiela PR et al. Cell confluence-induced activation of signal transducer and activator of transcription-3 (Stat3) triggers epithelial dome formation via augmentation of sodium hydrogen exchanger-3 (NHE3) expression. The Journal of biological chemistry. 2007;282:9883-94
29. Nishimura M, Naito S. Tissue-specific mRNA expression profiles of human solute carrier transporter superfamilies. Drug metabolism and pharmacokinetics. 2008;23:22-44
30. Consortium GT. The Genotype-Tissue Expression (GTEx) project. Nature genetics. 2013;45:580-5
31. Bergwitz C, Miyamoto KI. Hereditary hypophosphatemic rickets with hypercalciuria: pathophysiology, clinical presentation, diagnosis and therapy. Pflugers Archiv : European journal of physiology. 2018
32. Hernando N, Deliot N, Gisler SM, Lederer E, Weinman EJ, Biber J et al. PDZ-domain interactions and apical expression of type IIa Na/P(i) cotransporters. Proceedings of the National Academy of Sciences of the United States of America. 2002;99:11957-62
33. Mahon MJ, Cole JA, Lederer ED, Segre GV. Na+/H+ exchanger-regulatory factor 1 mediates inhibition of phosphate transport by parathyroid hormone and second messengers by acting at multiple sites in opossum kidney cells. Molecular endocrinology. 2003;17:2355-64
34. Clark BJ, Murray RD, Salyer SA, Tyagi SC, Arumugam C, Khundmiri SJ et al. Protein-DNA Interactions at the Opossum Npt2a Promoter are Dependent upon NHERF-1. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2016;39:1-12
35. Karim Z, Gerard B, Bakouh N, Alili R, Leroy C, Beck L et al. NHERF1 mutations and responsiveness of renal parathyroid hormone. The New England journal of medicine. 2008;359:1128-35
36. Courbebaisse M, Leroy C, Bakouh N, Salaun C, Beck L, Grandchamp B et al. A new human NHERF1 mutation decreases renal phosphate transporter NPT2a expression by a PTH-independent mechanism. PloS one. 2012;7:e34764

37. Kuro-o M. A potential link between phosphate and aging--lessons from Klotho-deficient mice. Mechanisms of ageing and development. 2010;131:270-5
38. Evans KD, Hewett TA, Clayton CJ, Krubitzer LA, Griffey SM. Normal organ weights, serum chemistry, hematology, and cecal and nasopharyngeal bacterial cultures in the gray short-tailed opossum (Monodelphis domestica). Journal of the American Association for Laboratory Animal Science : JAALAS. 2010;49:401-6

Figure legends

Figure 1 Cloning of *Didelphis virginiana* (OK cells) NaPi-IIc

The amino acid sequence of *Didelphis virginiana* NaPi-IIc (oNaPi-IIc) as deduced from the cDNA. Two variants (variant1 and variant2) of *D. virginiana* NaPi-IIc were identified. Predicted transmembrane regions, numbered 1–12, are underlined. *D. virginiana* NaPi-IIc variant1 amino acid residues that were common to *D. virginiana* (variant2), *M. domestica*, human, and mouse NaPi-IIc are filled in black.

Figure 2 Evaluation of oNaPi-IIc in OK cells

(A) Levels of oNaPi-IIc mRNA on days 2, 3, 4, and 6 after OK cell seeding. RT-PCR was used for mRNA analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was amplified as an internal control. Results are expressed as means ± SE (n=3), *$p < 0.05$ *vs* 2 days. (B, C) Production of oNaPi-IIc antibodies. The various antibodies (N-1, N-2, and C-1) prepared are shown in panel B. Antibodies were produced at two sites (N-1, N-2) on the N-terminal side and one site (C-1) on the C-terminal side. oNaPi-IIc antibodies were evaluated by Western blotting. Proteins were isolated as described previously [21]. Detection of oNaPi-2c was carried out using total cell lysate on day 6 after OK cell seeding. FLAG-oNaPi-IIc in OK cells was used as an oNaPi-IIc control. (D) Peptide competition assay. Total cell lysates from OK cells transfected with FLAG-oNaPi-IIc were separated by SDS-PAGE, and the blotted membrane was probed with anti-oNaPi-IIc antibodies (N-1), which was preincubated with

(+) or without (-) oNaPi-IIc N-terminus peptide (pos: 22-40) overnight. (E) Protein size detection of oNaPi-IIc. OK cells transfected with empty vector (pCMV-Tag2A vec.), FLAG - human NaPi-IIa, FLAG - human, mouse, and opossum NaPi-IIc were analyzed by Western blotting and the size of NaPi-IIc was determined. (F) Expression of oNaPi-IIa (NaPi-4) and oNaPi-IIc protein in OK cells. Western blotting was used for protein expression analysis. Actin was used as an internal standard. (G) Localization of oNaPi-IIc in transiently transfected OK cells were analyzed by immunofluorescence. OK cells transfected with FLAG-oNaPi-IIc were stained with antibodies against oNaPi-IIc (N-1) and F-actin. In merged images, oNaPi-IIc is shown in green, F-actin is shown in red, and the overlay of both signals is shown in yellow.

Figure 3 Characterization of oNaPi-IIc in Xenopus oocytes

(A) Oocytes injected with either water (def: defolliculation) or oNaPi-IIc cRNA were assayed after 2 days for uptake of Pi (100 μM) in 96 mM NaCl medium. Means ± SE (n=5). (B) Ion dependence of Pi transport in oocytes expressing oNaPi-IIc. The uptake of Pi was measured in standard uptake solution (Na), Na^+-free uptake solution in which Na^+ was replaced with choline (choline) and in Cl^--free uptake solution in which Cl^- was replaced with gluconate (gluconate). Means ± SE (n=5). (C) pH dependence of oNaPi-IIc-mediated Pi uptake. oNaPi-IIc-mediated uptake of Pi (100 μM) was measured in the standard uptake solution at various pH values. Means ± SE (n=5). (D) The Pi concentration dependence of oNaPi-IIc-mediated Pi uptake. oNaPi-IIc-mediated Pi uptake was measured at 10, 30, 100, 300, 600, and 1000 μM Pi in standard uptake solution. Means ± SE (n=5). $^{\#}p < 0.05$ *vs* def, $*p < 0.05$ *vs* Na^+, $^{\blacklozenge}p < 0.05$ *vs* ND 100, $^{a}p < 0.05$ *vs* pH 8.5.

Figure 4 Effect of oNaPi-IIc siRNA on membrane proteins in OK cells

(A) Schematic of the experiment schedule. OK cells were grown for 24 h and transfected with each siRNA construct at day 1. Total cell lysates or total mRNAs were prepared on day 6 after seeding. (B) Levels of oNaPi-IIc mRNA were assessed by RT-PCR analysis. GAPDH mRNA was amplified as an internal control. Means ± SE (n=6), *p < 0.05 *vs* oNaPi-IIc scramb. (C-D) Expression of various membrane proteins (oNaPi-IIc, oNaPi-IIa, NHE3, Megalin, Na^+/K^+-ATPase, Villin, and NHERF-1) in OK cells was analyzed by Western blot analysis. Actin was used as an internal control. (E) Densitometric analysis of oNaPi-IIa, NHE3, Na^+/K^+-ATPase, and NHERF-1. Band density was normalized against that of actin. Values are means ± SE (n=4), $^{\#}p < 0.05$ *vs* non transfected cells, $^{\#\#}p < 0.01$ *vs* non transfected cells, $*p < 0.05$ *vs* oNaPi-IIc scramb. $**p < 0.01$ *vs* oNaPi-IIc scramb. (F) Levels of oNaPi-IIa, NHE3, and Megalin mRNA were assessed by RT-PCR analysis. GAPDH mRNA was amplified as an internal control. Means ± SE (n=6), $*p < 0.05$ *vs* oNaPi-IIc scramb. (G) Pi uptake was assessed in OK cells transfected with oNaPi-IIa and oNaPi-IIc siRNA. Results are expressed as means ± SE (n=6) of uptake values. $*p<0.05$ *vs* each control siRNA.

Figure 5 Effect of siRNAs on dome formation in OK cells

OK cells (1.4×10^5 cells/well) were plated in 6-well plates and grown. Cells were monitored using BioStation CT and the number of domes that formed in the wells measured. (A, B) The target genes were knocked down with oNaPi-IIa siRNA or oNaPi-IIc siRNA. (A) Dome formation on day 5 after OK cell seeding. (B) The number of domes in wells (culture area: 1.8 cm^2/well) was counted on days 4, 5, 6, and 7 after seeding. $*p < 0.05$ *vs* each control siRNA. (C) Transport inhibitor assay in dome formation. Cells were treated with 1 or 2 mM sodium phosphonoformate tribasic hexahydrate (PFA; NaPi-II transport inhibitor; Sigma-Aldrich, P6801) at day 4. The number of domes in wells (culture area: 1.8 cm^2/well) was counted on days 4, 5, 6, and 7 after seeding. Means ± SE (n=4).

Figure 6 NaPi-IIa and NaPi-IIc protein in the kidney of Npt2c knockout mice and human adult kidney

(A) Immunoblotting of NaPi-IIa, NaPi-IIc, NHERF-1, and PDZK1 proteins in brush border membrane vesicles isolated from the kidney of control and $Npt2c^{-/-}$ mice (n=6). Actin was used as an internal control. (B) Immunoblotting analysis of NaPi-IIa and NaPi-IIc proteins in whole homogenate of human adult kidneys (No. 111 and No. 112) and positive control (OK cells transfected with FLAG-human NaPi-IIa and NaPi-IIc). Specific antibodies were used for the detection of human NaPi-IIa and NaPi-IIc, as described in the Materials and Methods. NaPi-IIa and NaPi-IIc positive bands are indicated as black arrows.

Table 1 Primer for 5' Race

Gene	Sense (5'-3')	Antisense (5'-3')	Reference
oNaPi-IIc	ACTAAGCTTGAAGAGGGGGAAGCAGATCC	ACTGATATCTCACAGTCTCTGGGAGGCCAG	XM_007475445

Table 2 siRNAs

Gene		Sense (5'-3')	Antisense (5'-3')	Reference
oNaPi-IIc	scrambled (control)	AUACGUUACGUUUUACGCCdTdT	GGCGUAAAACGUAACGUAUdTdT	-
	no.1	CUCCUCAAAGUUGUUGACUdTdT	AGUCAACAACUUUGAGGAGdTdT	-
	no.2	CUGUGAAGAACAUCAGUGAAUGCdAdG	CUGCAUUCACUGAUGUUCUUCACAGG	
Negtive control siRNA				-
oNaPi-IIa	no.1	UCACCAACACCAUAGUGGCAUUAAU	AUUAAUGCCACUAUGGUGUUGGUGA	L26308 (NCBI Nucleotide)
	no.2	GCUACAGUCCAUGACUGCUUUAACU	AGUUAAAGCAGUCAUGGACUGUAGC	

Table 3 Primers for RT-PCR

Gene	Sense (5'-3')	Antisense (5'-3')	product size (bp)	Reference
oNaPi-IIa	GCTGGCTCCCTTACCCTACTG	GATGACTGTCTGGACCACCTTG	102	L26308 (NCBI Nucleotide)
oNaPi-IIc	TTCTCAGTTCTGCCTTCCA	TCTGTACCAGTACAGTGACCA	126	-
NHE3	AGTTGCTTATGCCCTGGTTG	ACCAAGGGTTTGATGGTCAG	129	L42522.1
Megalin	AGGCTCCCTTCTGCCATCTCTTC	GCAGAATCTGGTCCAAAACCTGACAC	144	Endocrinology. 2009 Feb;150(2):871-8.
GAPDH	GGTCATACCTGAGCTGAACG	TCTGAAGCTTGCTTCACCAC	144	KC862381.1
oNaPi-IIc variant1&2	CAAGAGAGGAATGTCACGAC	GGAATTCAGCGTTGATGACC	214, 154	-
oNaPi-IIc variant1	GTGCTACCCTTACTGACCTG	GGAATTCAGCGTTGATGACC	156	-

NHE3: Na+/H+ exchanger 3, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

Figure 1

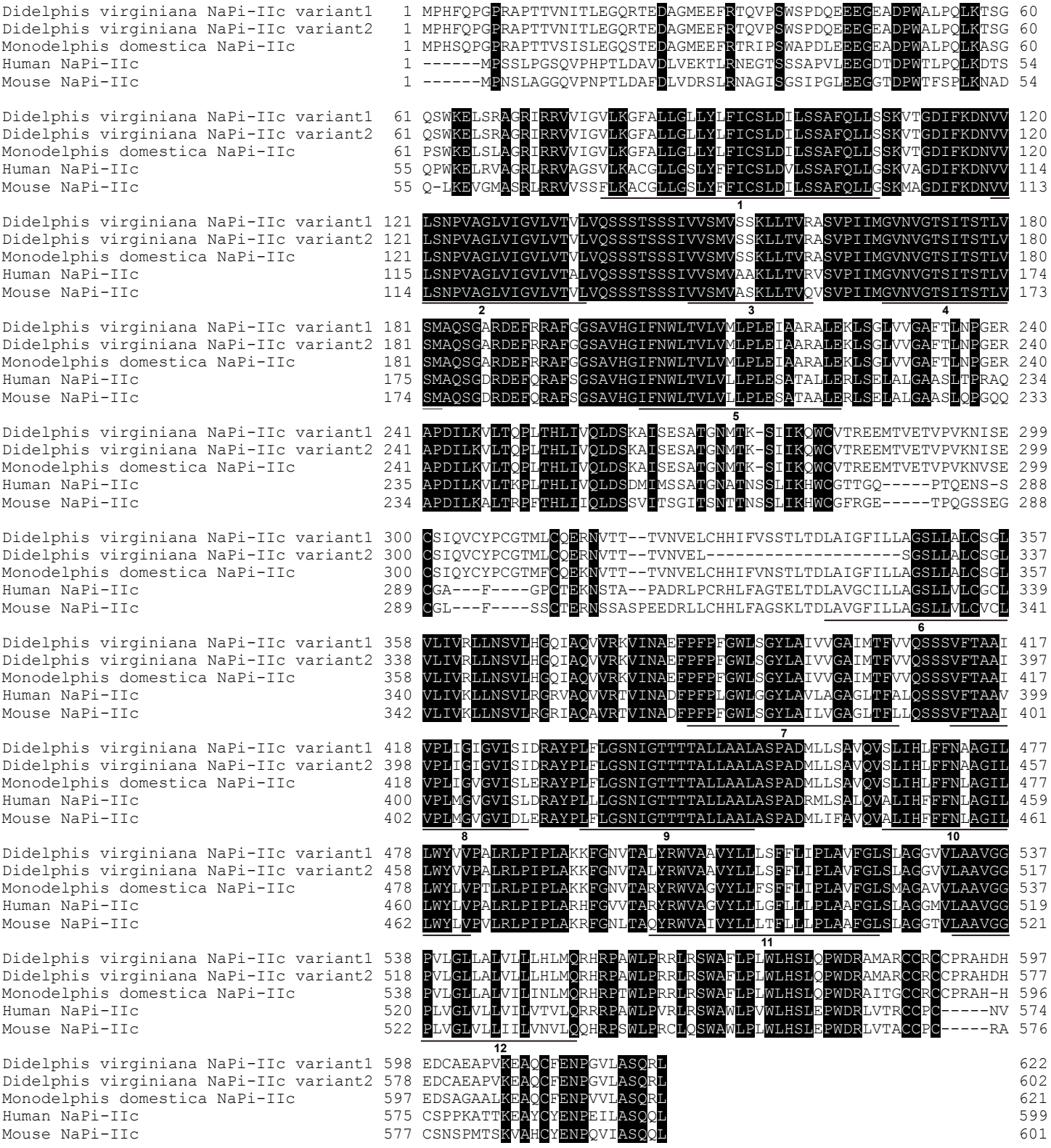
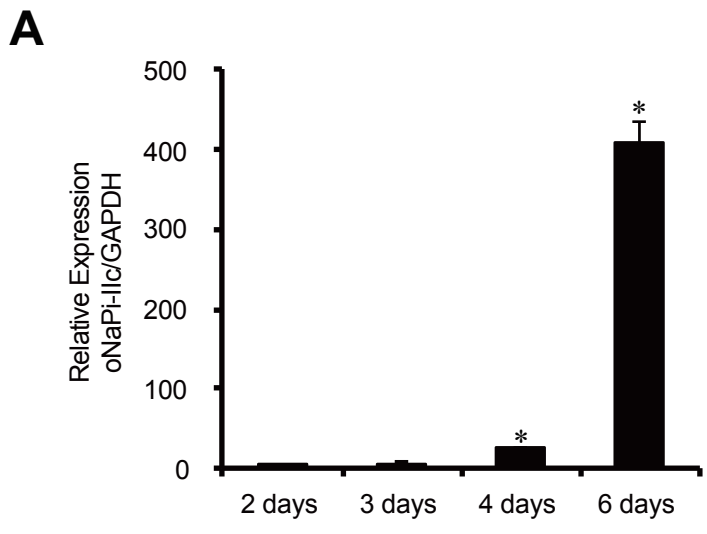
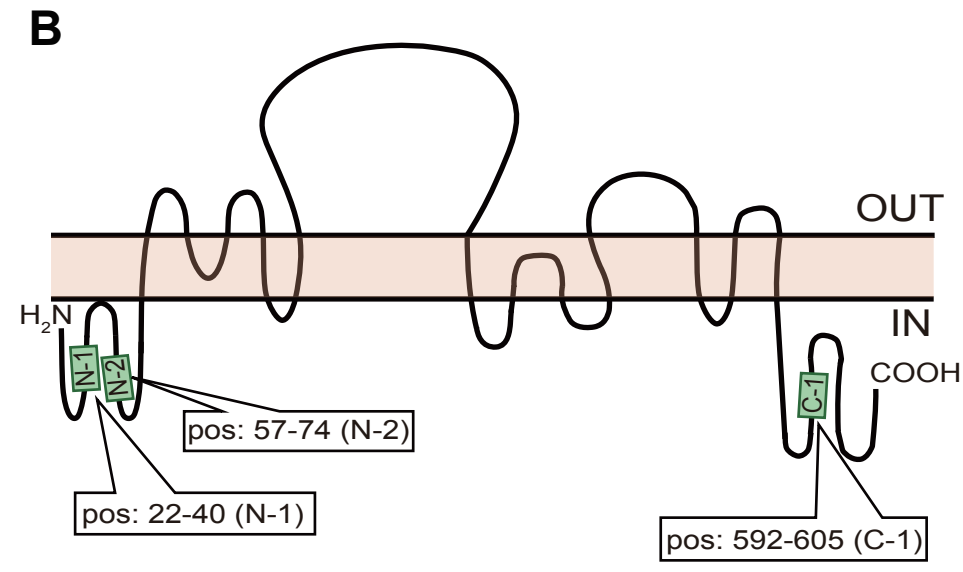
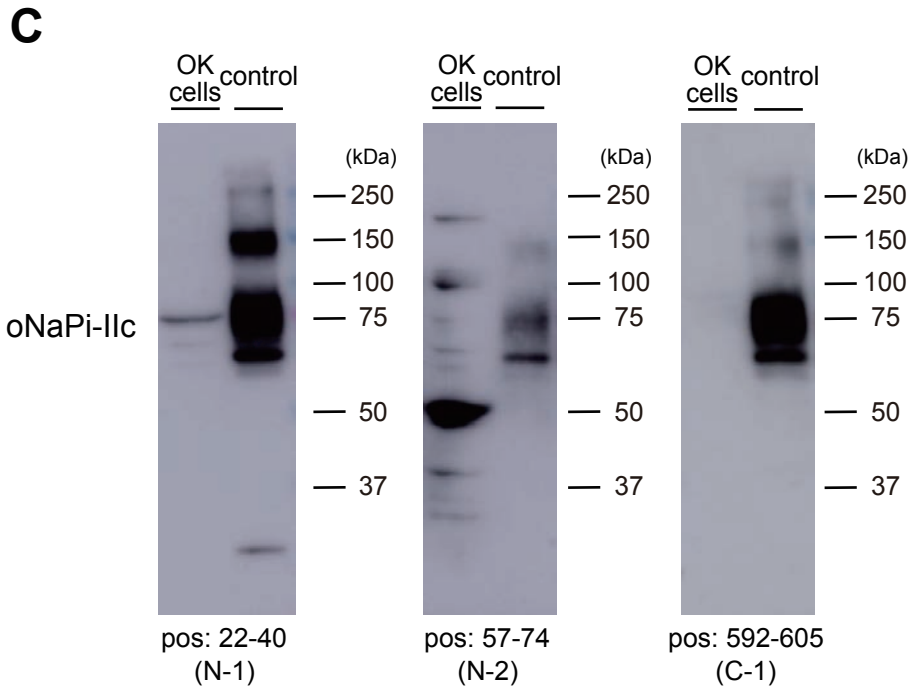
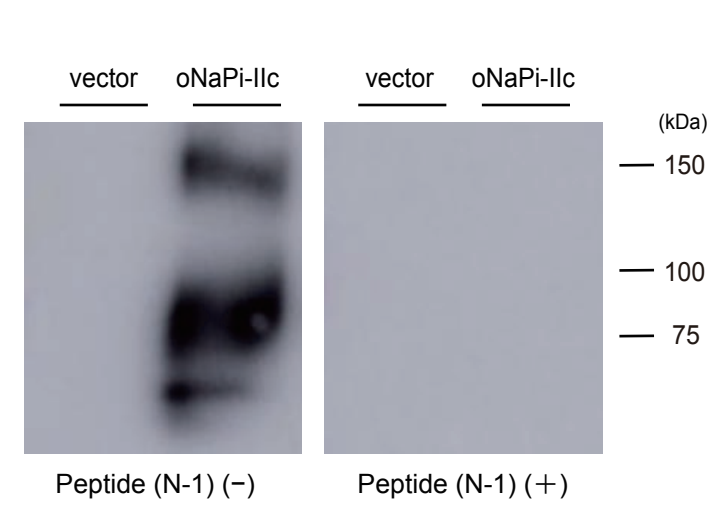

Didelphis virginiana NaPi-IIc variant1
Didelphis virginiana NaPi-IIc variant2
Monodelphis domestica NaPi-IIc
Human NaPi-IIc
Mouse NaPi-IIc
1
2
3
4
5
6
7
8
9
10
11
12

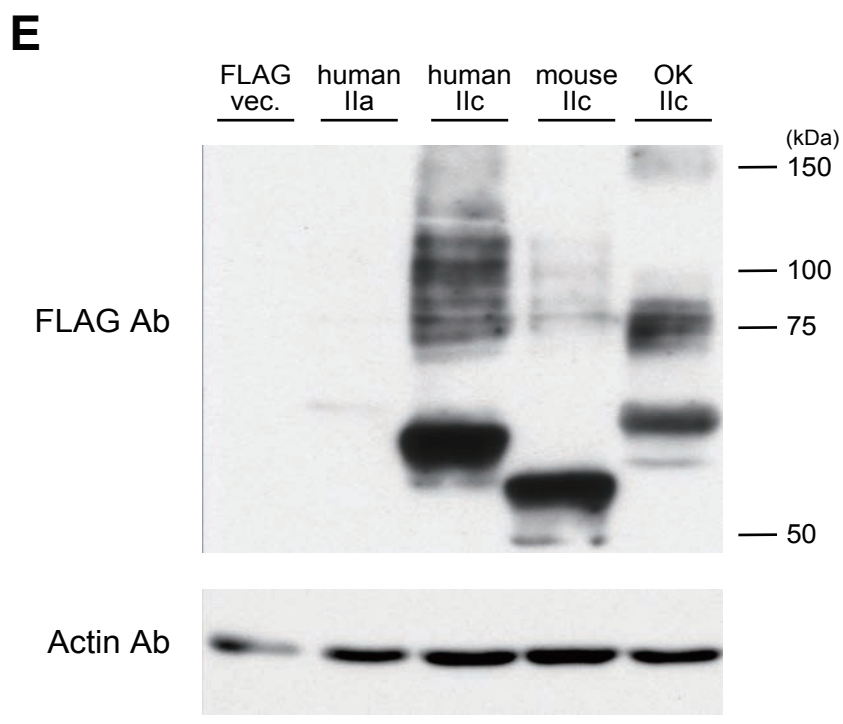
Figure 2


A
Relative Expression
oNaPi-IIc/GAPDH
500
400
300
200
100
0
*
*
2 days
3 days
4 days
6 days


B
OUT
IN
H2N
COOH
N-1
N-2
C-1
pos: 57-74 (N-2)
pos: 22-40 (N-1)
pos: 592-605 (C-1)


C
OK cells
control
(kDa)
250
150
100
75
50
37
oNaPi-IIc
pos: 22-40
(N-1)
pos: 57-74
(N-2)
pos: 592-605
(C-1)


D
vector
oNaPi-IIc
vector
oNaPi-IIc
(kDa)
150
100
75
Peptide (N-1) (−)
Peptide (N-1) (＋)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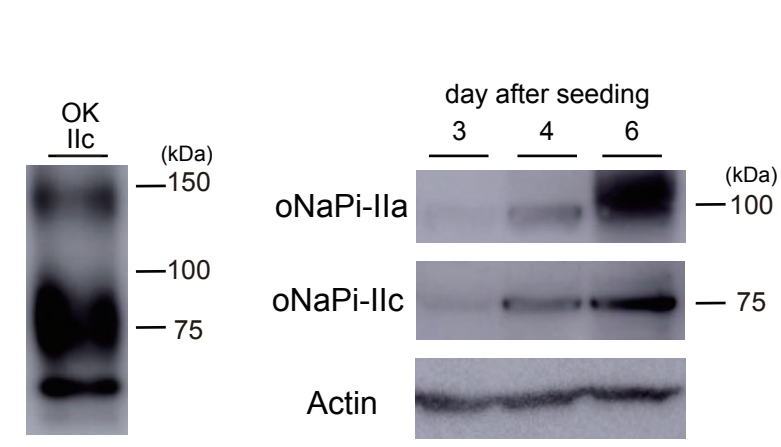
FLAG vec.
human IIa
human IIc
mouse IIc
OK IIc
(kDa)
150
100
75
50
FLAG Ab
Actin Ab


F
OK IIc
(kDa)
150
100
75
day after seeding
3
4
6
oNaPi-IIa
oNaPi-IIc
Actin
(kDa)
100
75

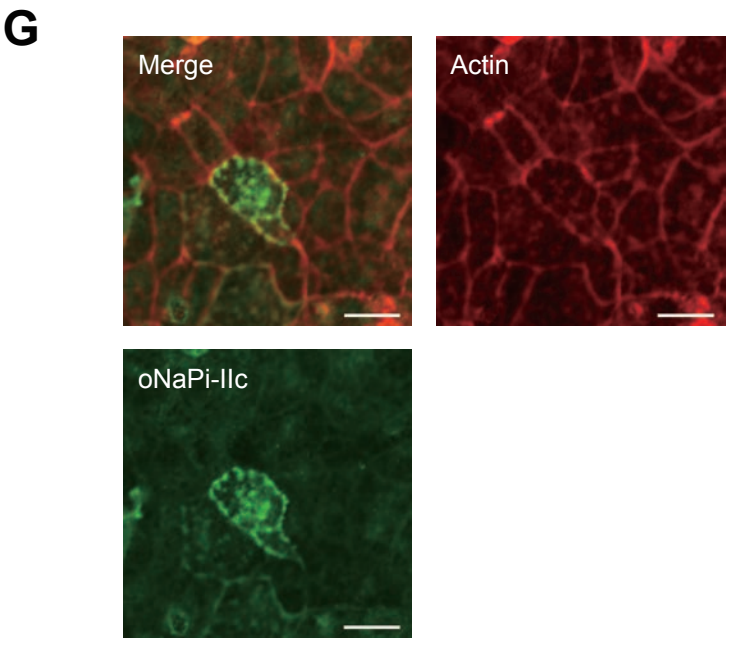

G
Merge
Actin
oNaPi-IIc

Figure 3

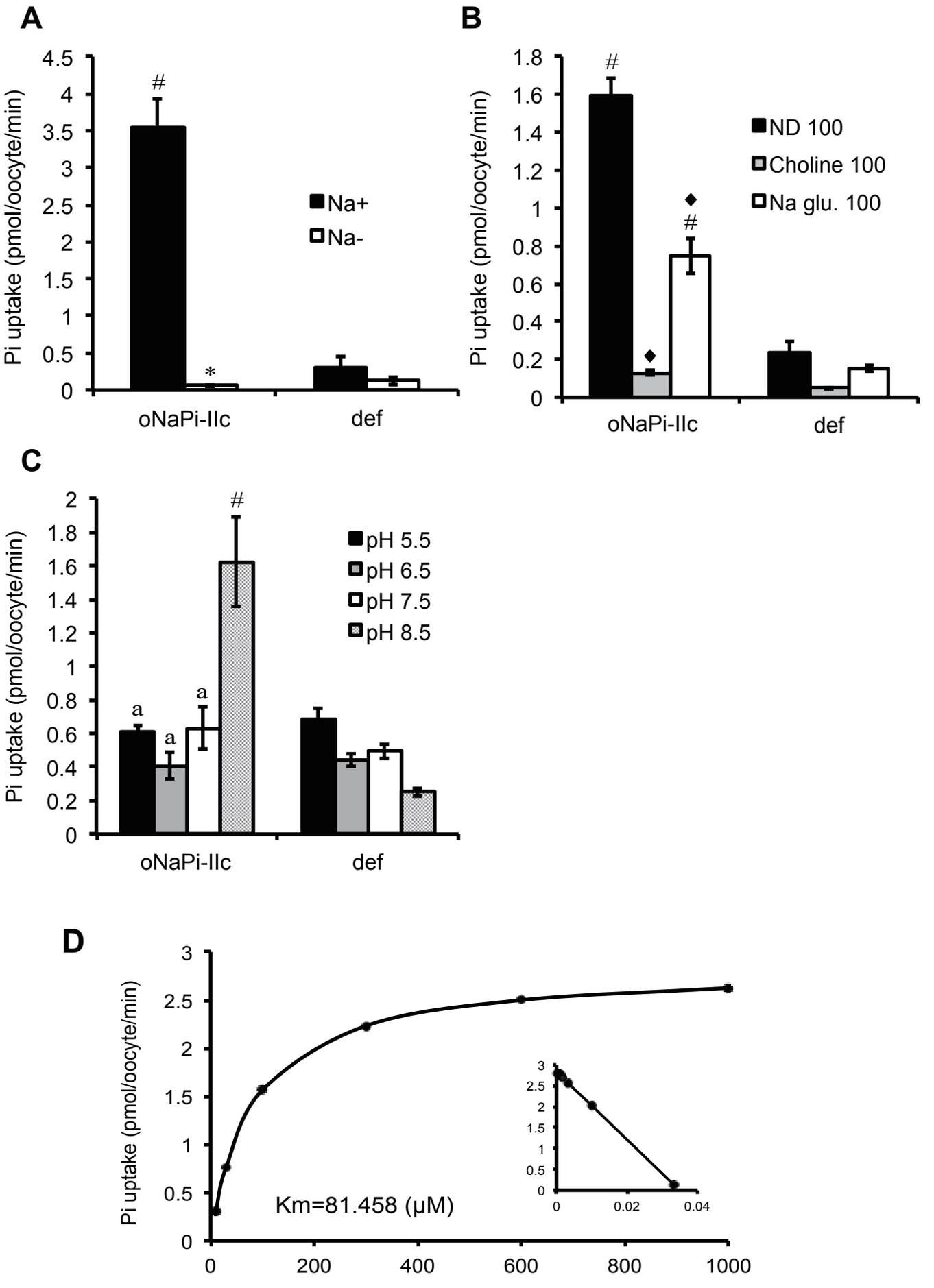
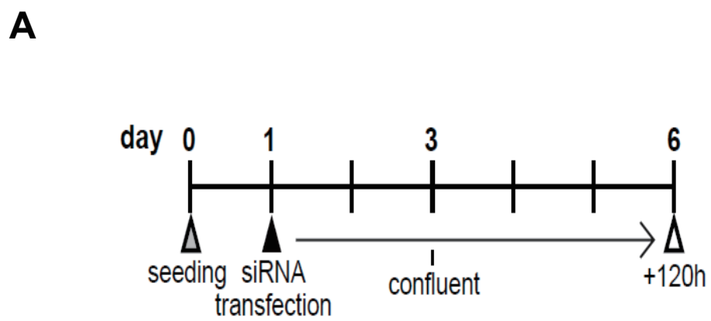

A
Pi uptake (pmol/oocyte/min)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
#
*
Na+
Na-
oNaPi-IIc
def
B
Pi uptake (pmol/oocyte/min)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
#
ND 100
Choline 100
Na glu. 100
oNaPi-IIc
def
C
Pi uptake (pmol/oocyte/min)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
#
pH 5.5
pH 6.5
pH 7.5
pH 8.5
oNaPi-IIc
def
D
Pi uptake (pmol/oocyte/min)
3
2.5
2
1.5
1
0.5
0
Km=81.458 (μM)
0
200
400
600
800
1000
3
2.5
2
1.5
1
0.5
0
0
0.02
0.04

Figure 4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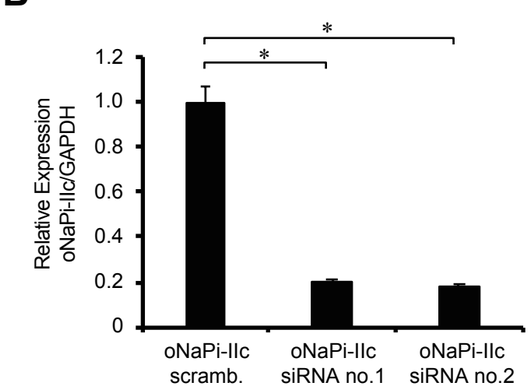
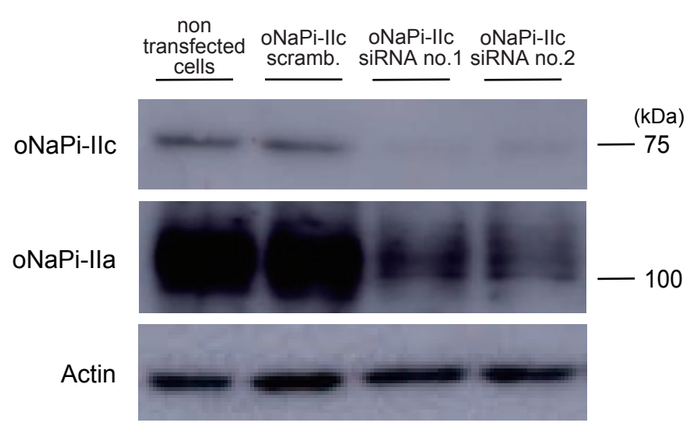
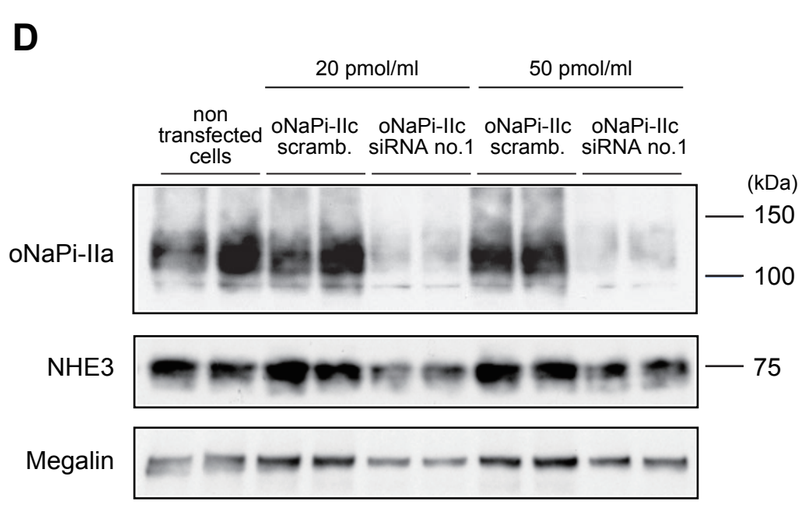
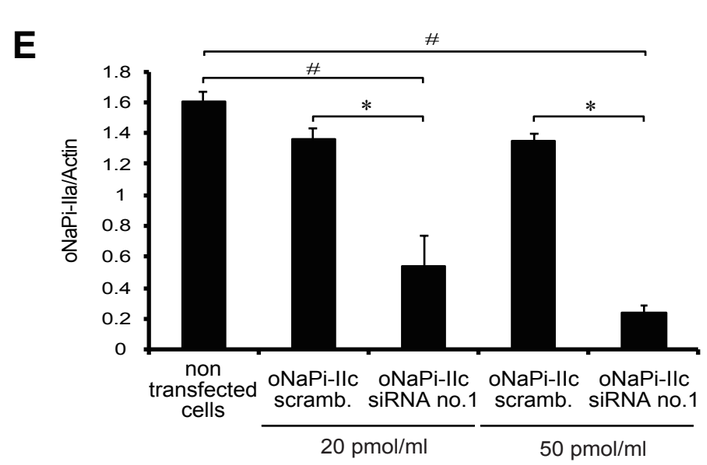
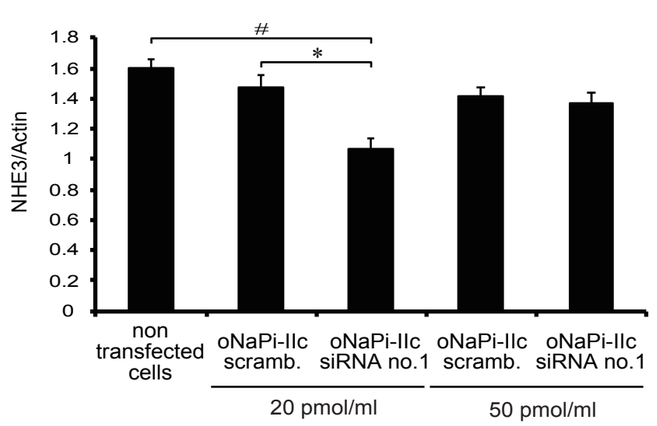
day 0
1
3
6
seeding
siRNA transfection
confluent
+120h

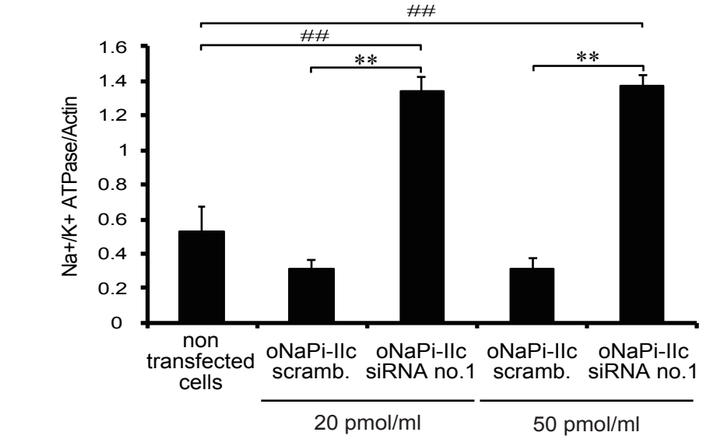

B
Relative Expression oNaPi-IIc/GAPDH
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
oNaPi-IIc siRNA no.2

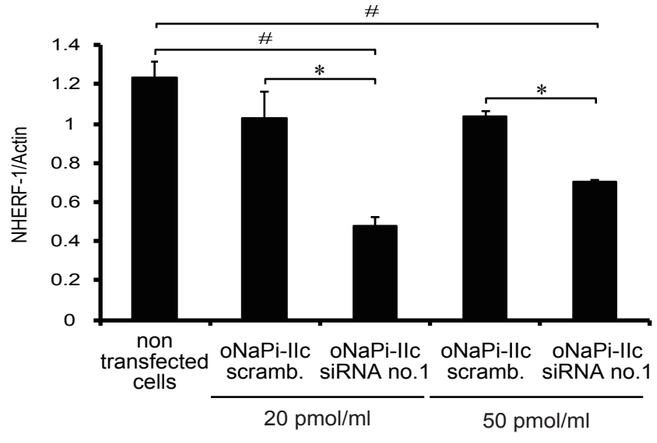

C
non transfected cells
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
oNaPi-IIc siRNA no.2
(kDa)
oNaPi-IIc
75
oNaPi-IIa
100
Actin

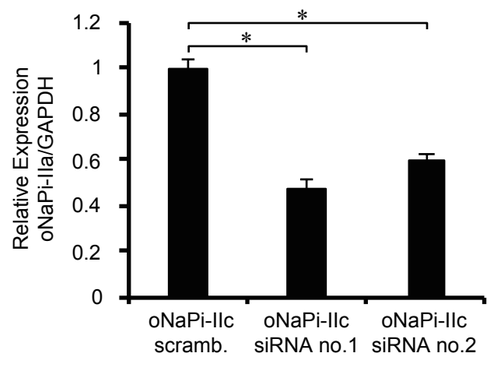

D
20 pmol/ml
50 pmol/ml
non transfected cells
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
(kDa)
oNaPi-IIa
150
100
NHE3
75
Megalin
Na+/K+ ATPase α1
100
75
Villin
100
NHERF-1
50
Actin

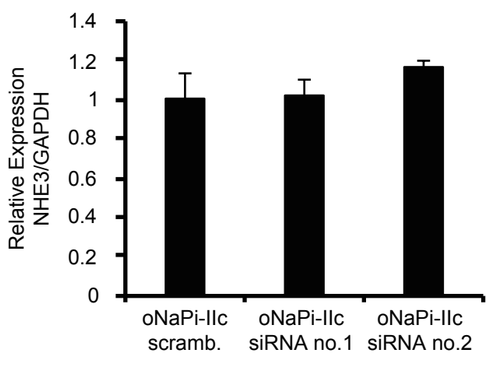

E
oNaPi-IIa/Actin
#
*
*
non transfected cells
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
20 pmol/ml
50 pmol/ml

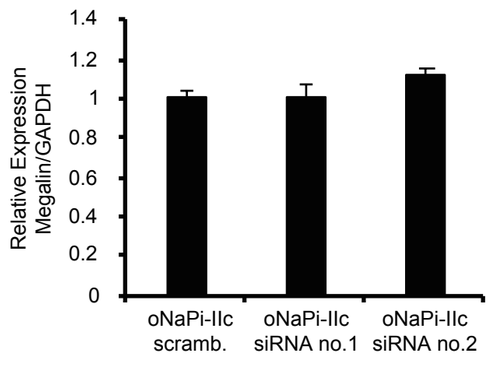

NHE3/Actin
#
*
non transfected cells
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
20 pmol/ml
50 pmol/ml


Na+/K+ ATPase/Actin
##
**
non transfected cells
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
20 pmol/ml
50 pmol/ml


NHERF-1/Actin
#
*
non transfected cells
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
20 pmol/ml
50 pmol/ml


F
Relative Expression oNaPi-IIa/GAPDH
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
oNaPi-IIc siRNA no.2


Relative Expression NHE3/GAPDH
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
oNaPi-IIc siRNA no.2


Relative Expression Megalin/GAPDH
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
oNaPi-IIc siRNA no.2

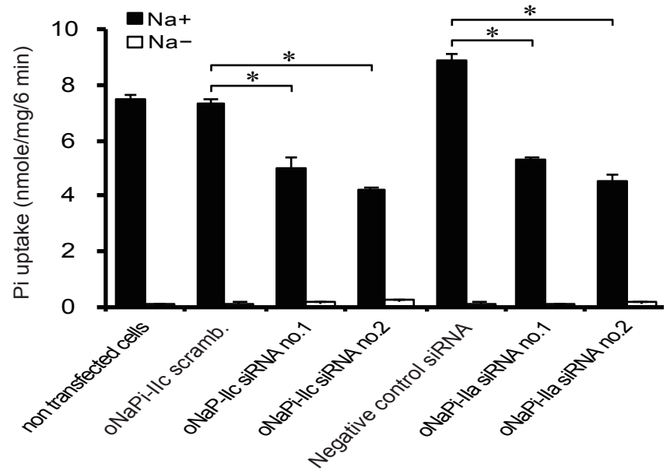

G
Na+
Na−
Pi uptake (nmole/mg/6 min)
non transfected cells
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
oNaPi-IIc siRNA no.2
Negative control siRNA
oNaPi-IIa siRNA no.1
oNaPi-IIa siRNA no.2

Figure 5

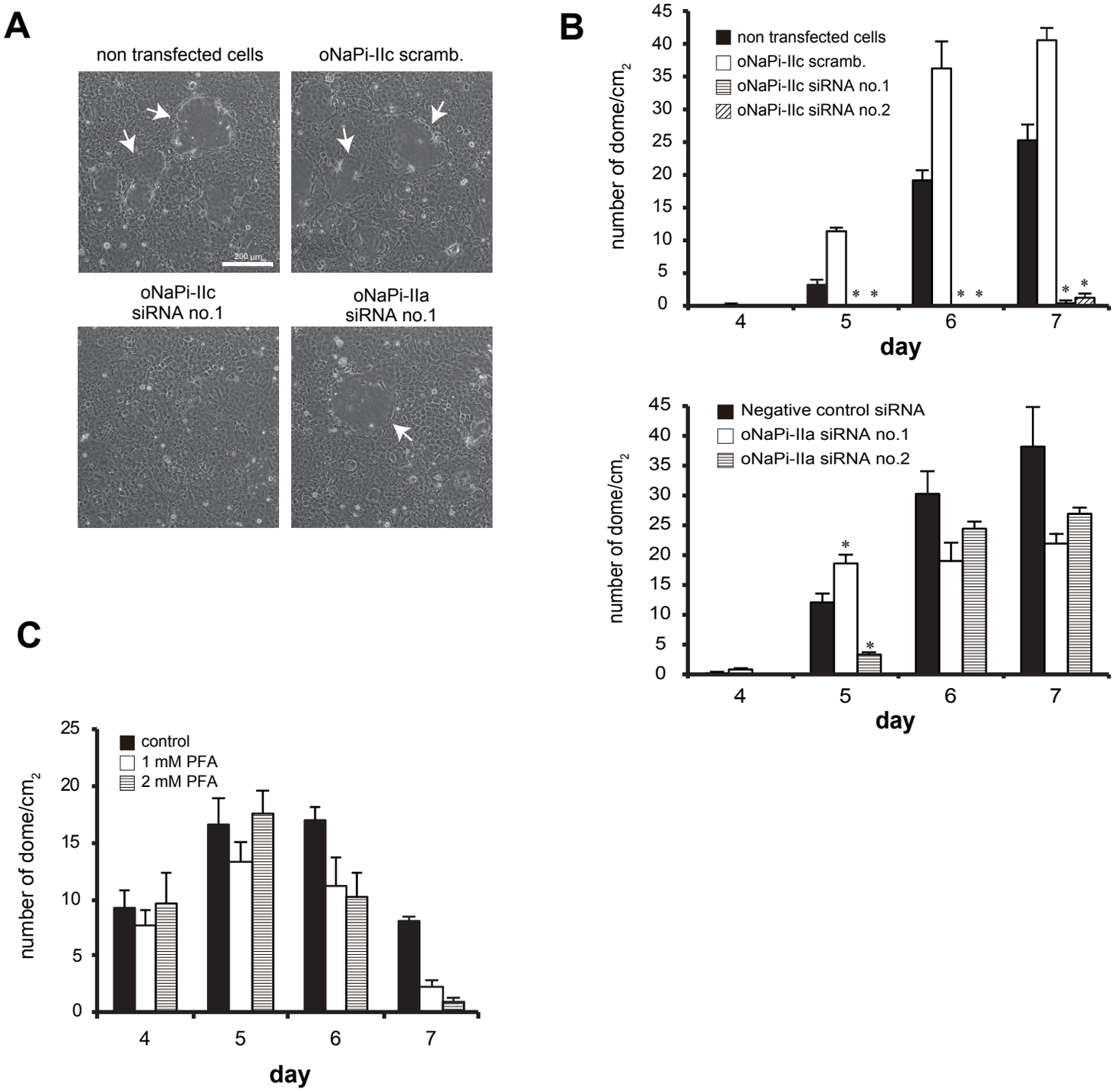
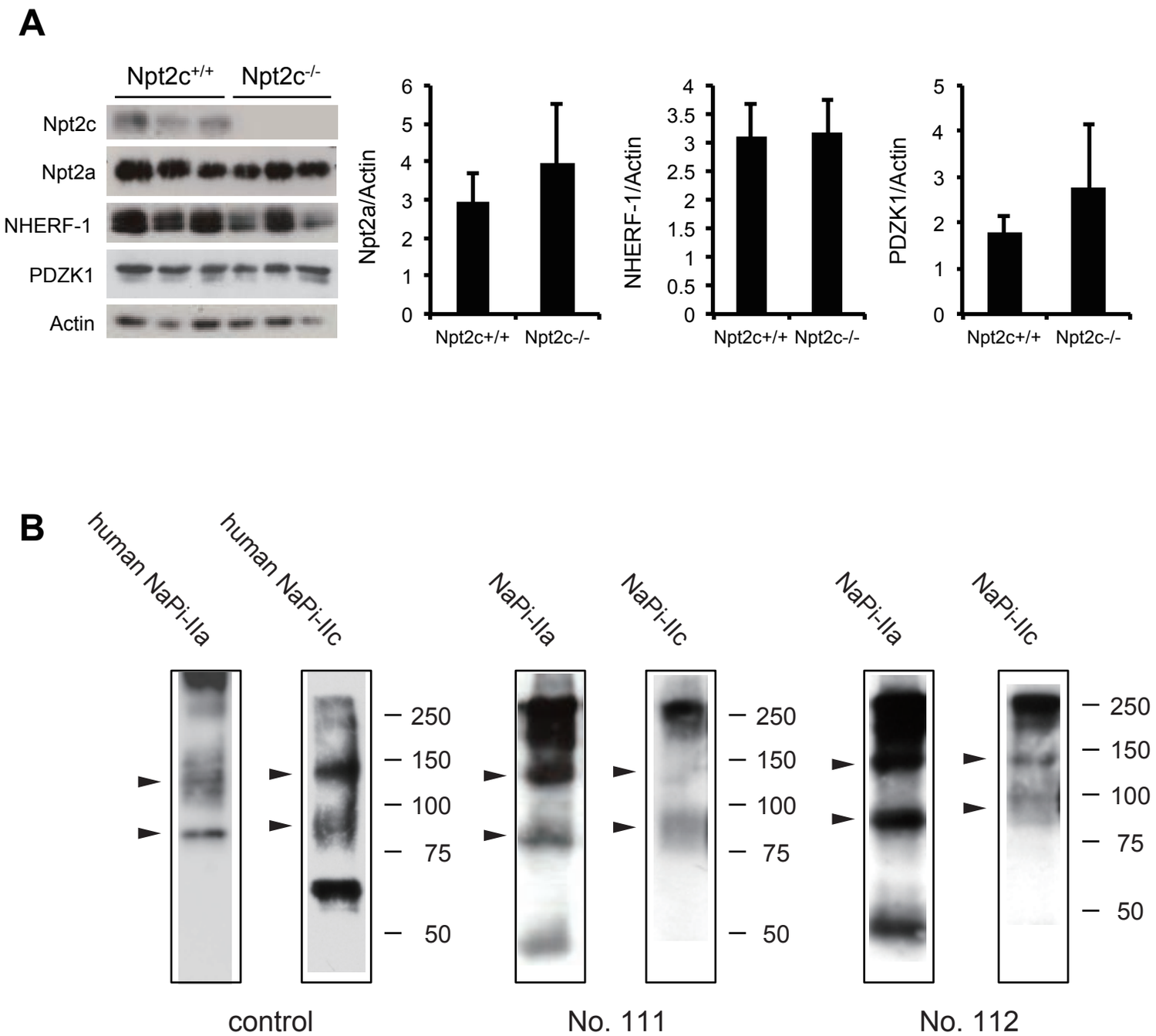

A
non transfected cells
oNaPi-IIc scramb.
200 µm
oNaPi-IIc
siRNA no.1
oNaPi-IIa
siRNA no.1
B
non transfected cells
oNaPi-IIc scramb.
oNaPi-IIc siRNA no.1
oNaPi-IIc siRNA no.2
number of dome/cm2
day
Negative control siRNA
oNaPi-IIa siRNA no.1
oNaPi-IIa siRNA no.2
number of dome/cm2
day
C
control
1 mM PFA
2 mM PFA
number of dome/cm2
day

Figure 6


A
Npt2c+/+
Npt2c-/-
Npt2c
Npt2a
NHERF-1
PDZK1
Actin
Npt2a/Actin
NHERF-1/Actin
PDZK1/Actin
Npt2c+/+
Npt2c-/-
B
human NaPi-IIa
human NaPi-IIc
NaPi-IIa
NaPi-IIc
NaPi-IIa
NaPi-IIc
250
150
100
75
50
control
No. 111
No. 112

Supplemental Figure 1